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Optimal Induction of Hepatitis C Virus Envelope-Specific Immunity by Bicistronic Plasmid DNA Inoculation with the Granulocyte-Macrophage Colony-Stimulating Factor Gene

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In this study, we have constructed various DNA vaccine vectors that carried hepatitis C virus (HCV) envelope genes without and with the granulocyte-macrophage colony-stimulating factor (GM-CSF) gene in several different ways. In Buffalo rats that received plasmids carrying the HCV envelope genes, which encode envelope proteins E1 and E2, both antibody and lymphoproliferative responses against these proteins were induced. These responses were greatly enhanced by the codelivery of the GM-CSF gene. In particular, inoculation with a bicistronic plasmid that independently expressed the GM-CSF gene and the envelope genes in the same construct generated the highest antibody titers and significantly increased lymphoproliferative responses against these proteins. Moreover, strong antibody responses to homologous and heterologous hypervariable region 1 peptides were elicited in the immunized rats.

Hepatitis C virus (HCV) has been identified as a major causative agent of posttransfusion and sporadic non-A, non-B hepatitis (2, 13). More than 70% of HCV infections are persistent and eventually lead to liver cirrhosis and hepatocellular carcinoma (28). To date, the only treatment for chronic HCV infection is alpha interferon therapy. However, long-term responses to this therapy occur in only 10 to 30% of patients (20, 25). Therefore, the development of a vaccine to prevent HCV infection is of the greatest urgency. HCV has a 9.5-kb positive-strand RNA genome that encodes a single polypeptide. The polypeptide is processed by cellular and viral proteinases to produce both the structural and the nonstructural HCV proteins (4, 10, 30). Based on data that was derived from clinical and experimental studies of humans and chimpanzees, it has been suggested that both humoral and cellular immune responses to HCV proteins can be generated (8, 11, 24, 26). It has been shown that HCV envelope proteins 1 and 2 appear to be key viral antigens for the induction of protective immunity in experimental chimpanzees (3). Recently, DNA vaccine approaches have been applied to generate immunity to HCV proteins. The expression of the HCV core and E2 proteins resulted in the generation of HCV antigen-specific immune responses (14, 19, 21, 33).

The use of cytokines to modulate immune responses in DNA immunization is being actively investigated. Granulocyte-macrophage colony-stimulating factor (GM-CSF), a hematopoietic growth factor, has been widely used as a molecular adjuvant to induce immunity. It has been shown that idiotype–GM-CSF fusion proteins are effective vaccines for lymphoma, without the need for another adjuvant (32). In addition, the intramuscular inoculation of the GM-CSF gene together with plasmids carrying viral genes, such as those encoding the glycoprotein of rabies virus and VP1 of encephalomyocarditis virus, increased antigen-specific immune responses and protective immunity (31, 36). Other cytokines such as interleukin-2, interleukin-12, and gamma interferon have also been shown to enhance the immune responses to coadministered antigens (5, 12, 37). These reports suggest that the local expression of relevant cytokine genes can affect the microenvironment, which allows for immune responses to be elicited by the coadministered antigens.

In this study, we compared the levels of immune responses induced by HCV E1 and E2 DNA-based immunization without and with various forms of the GM-CSF gene in Buffalo rats. Our result demonstrated that HCV envelope-specific immune responses were significantly enhanced by the codelivery of the GM-CSF gene. The coexpression of the GM-CSF and HCV envelope proteins from a bicistronic vector most effectively generated envelope-specific antibodies and lymphoproliferative responses. Furthermore, cross-reactive antibodies directed against HVR1 peptides of homologous and heterologous strains were generated by these procedures.

Construction and identification of various expression plasmids. pTV2 was constructed from PUC19 as an expression vector for DNA vaccine. This eukaryotic expression vector contains the cytomegalovirus early promoter/enhancer sequence, the simian virus 40 (SV40) replication origin sequence, the adenovirus tripartite leader, and the SV40 polyadenylation sequence. To construct HCV envelope-based DNA vaccine vectors, we replaced the signal sequences of the E1 and the E2 proteins with that of herpes simplex virus type 1 glycoprotein D (gD). This signal sequence has been shown to facilitate the efficient expression and secretion of human immunodeficiency virus type 1 gp160 (1). In addition, C-terminal hydrophobic regions of envelope proteins were truncated to maximize the secretion of these proteins. To construct pSK-s, a PCR fragment that contained a signal sequence of herpes simplex virus type 1 gD (s; amino acid residues 1 to 34) was inserted into pBluescript SK(+) (Stratagene). HCV DNA fragments that encoded amino acid residues 192 to 364 and 384 to 719, which were designated E1t and E2t, respectively, of type 1b (Korean isolate) were amplified by PCR using E1S (5′-CCA GCT TCC AGA TCT GAA GCG CGT AAC-3′), E1AS (5′-GCC GAA TTC TAC ACC ATG GAA TAG TAG-3′), E2S (5′-CCA TAT GCG AGA TCT AGG AGG AAC G-3′), and E2AS

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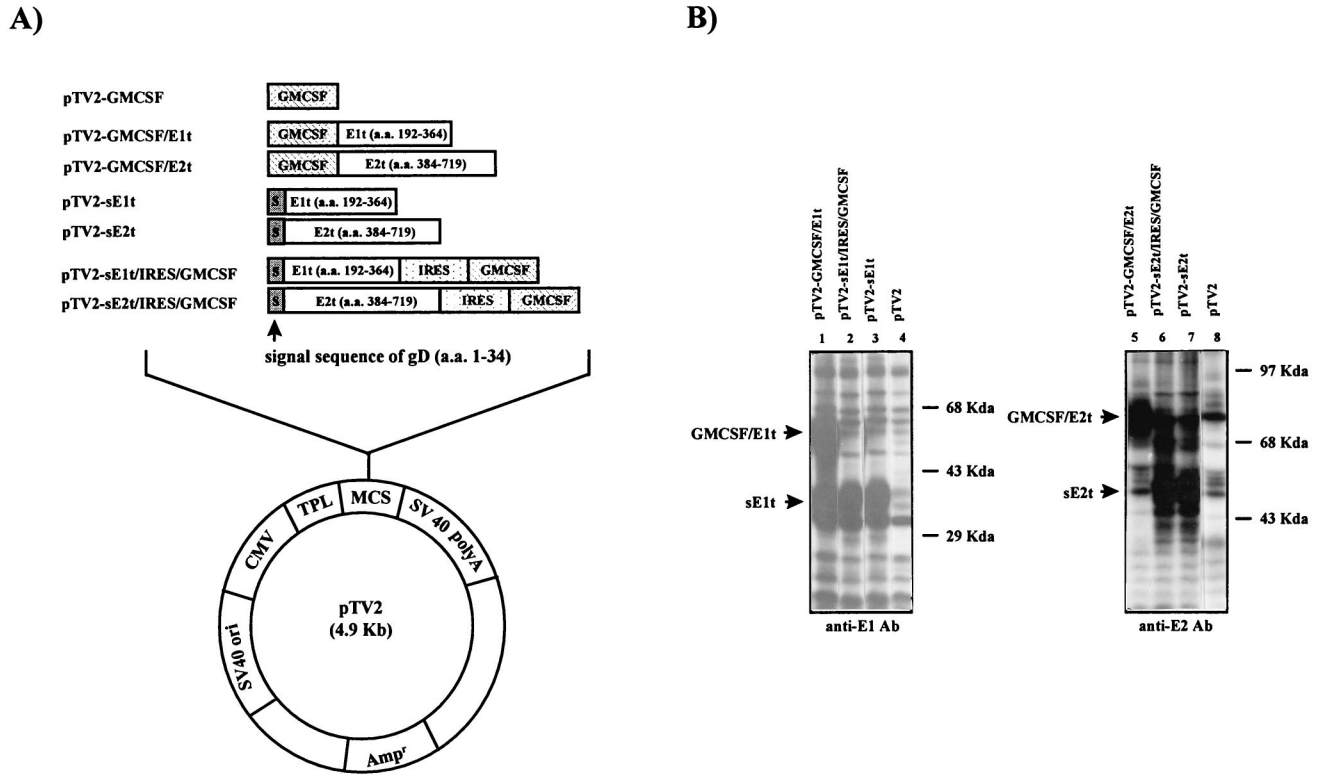

FIG. 1. (NEN Life Science Products) Schematic diagram of expression plasmids used in DNA immunization. All constructs were based on pTV2, represented at the bottom. pTV2-sE1t and pTV2-sE2t were designed to express the C-terminal-truncated E1t and E2t genes, respectively, which encompass the indicated regions, fused with the DNA encoding the signal sequence of gD. pTV2-GMCSF/E1t and pTV2-GMCSF/E2t were also designed to express the E1t and E2t genes fused with that of GM-CSF. pTV2-sE1t/IRES/GMCSF and pTV2-sE2t/IRES/GMCSF were each constructed to express both the HCV envelope and GM-CSF genes under the control of the cytomegalovirus (CMV) promoter and the internal ribosomal entry sequence (IRES), respectively, of encephalomyocarditis virus. pTV2-GMCSF was also made to express the GM-CSF gene alone. Both E1t and E2t genes are shown as open boxes, and the GM-CSF gene and IRES are shown as striped boxes and dotted boxes, respectively. Numbers in parentheses indicate the numbers of the amino acids encoded by the DNA fragment. (B) Identification of HCV envelope proteins. Transfected COS-7 cells were labeled with ^{35}S-Express label (NEN Life Science Products) and immunoprecipitated as previously described. Cell lysates were immunoprecipitated with either anti-E1 (lanes 1 to 4) or anti-E2 (lanes 5 to 8) monoclonal antibodies. Molecular mass markers and corresponding proteins are indicated at the right and left, respectively. Transfected plasmids are indicated at the top of each lane. SV40, simian virus 40; MCS, multiple cloning site; TPL, adenovirus tripartite leader.

(5′-GCG AAT TCT AAT ACT CCC ACC TGA TCG CA-3′) primers. The amplified products were digested with *Bgl*II and *Eco*RI and inserted downstream of pSK-s to produce pSK-sE1t and pSK-sE2t. The resulting plasmids were digested with *Xho*I and *Xba*I and then inserted into these same sites in pTV2 to generate eukaryotic expression vectors pTV2-sE1t and pTV2-sE2t (Fig. 1A). To investigate whether immune responses to HCV envelope proteins are modulated by the codelivery of the GM-CSF gene, we constructed several expression plasmids that carried HCV envelope genes in combination with the GM-CSF gene. A plasmid that expressed GM-CSF but not HCV sequences, pTV2-GMCSF, was constructed by inserting the murine GM-CSF gene for MFG–GM-CSF (6) into pTV2 (Fig. 1A). To construct plasmids that expressed GM-CSF and the HCV envelope proteins as fusion proteins, the GM-CSF gene was amplified by PCR from pTV2-GMCSF with the T7 universal primer and the GC598A primer (5′-CCG CCT CCC ATA TGG CAT TTT TGG ACT GG-3′). These sequences were replaced with hGH of either pSK-hGHE1t or pSK-hGHE2t to produce pSK-GMCSF/E1t and pSK-GMCSF/E2t, respectively. These plasmids were digested with *Eco*RI, and the GM-CSF and HCV sequences were inserted into pTV2 to generate pTV2-GMCSF/E1t and pTV2-GMCSF/E2t, respectively (Fig. 1A). To construct bicistronic plasmids, the GM-CSF gene was amplified by PCR with the GM-CSF N-terminal (GCN) primer (5′-GGA ACC ATG GGG ATG TGG CTG CAG AAT-3′) and the T7 universal primer. The amplified product was digested with *Nco*I and *Bam*HI and inserted downstream of pSK-IRES to generate pSK-IRES/GMCSF. The E1t and the E2t genes were individually inserted into pSK-IRES/GMCSF to produce pSK-sE1t/IRES/GMCSF and pSK-sE2t/IRES/GMCSF, respectively. These plasmids were digested with either *Xho*I and *Xba*I or *Asp*718 and *Xba*I, and the resulting fragments were inserted into pTV2 to generate pTV2-sE1t/IRES/GMCSF and pTV2-sE2t/IRES/GMCSF, respectively (Fig. 1A). These bicistronic plasmids were designed to coexpress each HCV envelope protein and GM-CSF from the same plasmid. To determine if these plasmids expressed immunologically relevant proteins, a transient transfection assay of COS-7 cells was performed as previously described (16, 17). As shown in Fig. 1B, the sE1t and the sE2t proteins were detected at estimated molecular masses of ~34 to 36 and ~49 to 51 kDa, respectively, when cell lysates were precipitated with either anti-E1 or anti-E2 monoclonal antibodies (lanes 2, 3, 6, and 7). It is likely that approximately 5% of the envelope proteins expressed from these constructs were secreted into culture supernatants (data not shown). In addition, the fusion proteins, GMCSF/E1t and GMCSF/E2t, were detected at molecular masses of ~59 to 65 and ~73 to 79 kDa, respectively (lanes 1 and 5). An ~31- to 33-kDa band that corresponded to

TABLE 1. Summary of plasmids injected into rats from different groups,[a] the end point titrations of antibodies in seroconverted rats,[b] and lymphoproliferative responses in immunized rats[c] to HCV E1 and E2 proteins

Plasmids (group)	No. of rats	Titer of:		Stimulation index with stimulant[d]:					
		Anti-E1	Anti-E2	hgh at:		hgh-E1t at:		hgh-E2t at:	
				1 μg/ml	10 μg/ml	1 μg/ml	10 μg/ml	1 μg/ml	10 μg/ml
pTV2 (I)	9	NT[e]	NT	0.8 ± 0.06	0.7 ± 0.05	3.1 ± 0.45	3.4 ± 0.49	0.9 ± 0.07	1.2 ± 0.21
pTV2-sE1t + pTV2-sE2t (II)	18	442	5,662	0.8 ± 0.13	0.9 ± 0.07	4.8 ± 1.79	9.8 ± 3.89	1.6 ± 0.84	4.4 ± 1.74
pTV2-sE1t + pTV2-sE2t + pTV2-GMCSF (III)	18	532	8,294	0.9 ± 0.13	0.9 ± 0.10	7.0 ± 3.23	13.0 ± 5.00	2.5 ± 0.57	6.3 ± 2.09
pTV2-GMCSF/E1t + pTV2-GMCSF/E2t (IV)	18	686	16,729	0.8 ± 0.13	0.9 ± 0.11	6.9 ± 1.52	11.9 ± 2.94	2.7 ± 1.39	7.0 ± 3.61
pTV2-sE1t/IRES/GMCSF + pTV2-sE2t/IRES/GMCSF (V)	18	1,615	71,647	0.9 ± 0.14	0.9 ± 0.10	7.4 ± 1.46	14.7 ± 6.12	4.6 ± 1.47	11.2 ± 1.81

[a] Female buffalo rats were injected three times with a total of 400 μg of plasmids into the anterior tibialis muscles at 8-week intervals. Two hundred micrograms of pTV2 plasmid was injected into the anterior tibialis muscle of each rat in group I, and 100 μg of each plasmid was injected into each rat in groups II, IV, and V. In group III, 80 μg of each of the pTV2-sE1t and pTV2-sE2t plasmids and 40 μg of the pTV2-GMCSF plasmid were injected.

[b] The anti-E1 and anti-E2 antibody titers were examined with pooled sera of each group of rats at 3 weeks after the last DNA injection (at week 19).

[c] Three weeks after the last DNA injections, each splenocyte obtained from immunized rats (six rats from each group) was used in proliferation assays.

[d] Values are mean stimulation indices for each rat ± standard deviations. Proliferation assays were carried out with triplicate wells.

[e] NT, not tested.

the E1t protein was also observed in pTV2-GMCSF/E1t-transfected cell lysates, presumably due to the cleavage of the junction region between the GM-CSF and the E1t proteins (lane 1). In contrast, a specific protein band was not detected in cell lysates that were transfected with control plasmid pTV2 (lanes 4 and 8). The culture supernatants of transfected COS-7 cells were assayed to determine the expression level of GM-CSF with a commercial GM-CSF enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems). All plasmids that encoded GM-CSF, including the fusion constructs, produced similar levels of GM-CSF, approximately 5.2 to 6.8 ng/ml (data not shown).

Antibody responses to HCV envelope proteins resulting from DNA immunizations with or without the codelivery of the GM-CSF gene. To elucidate the most effective GM-CSF codelivery method for the induction of immune responses to HCV envelope proteins, the various expression plasmids were injected into the anterior tibialis muscles of female Buffalo rats at 8-week intervals (Table 1). Four- to 6-week-old female Buffalo rats were purchased from Harlan Sprague-Dawley and were housed in the specific pathogen-free facility of the Pohang University of Science and Technology. Briefly, all the rats received intramuscular injections in the anterior tibialis muscle of 200 μg of plasmid DNA dissolved in 150 μl of sterile saline following pretreatment with bupivacaine-HCl (ASTRA) (35). This was followed by two booster immunizations with the same dose at weeks 8 and 16 after the initial DNA injection. Sera were collected by tail bleeding at selected time points and monitored for the presence of antibodies to HCV envelope 1 and 2 (E1 and E2) proteins by ELISA using the hghE1t and the hghE2t proteins that had been purified from recombinant Chinese hamster ovary (CHO) cell lines as specific antigens. Anti-E1 antibodies were initially detected at week 6 postinoculation. The seroconversion rate ranged from 6 to 11% for the different rat groups (Fig. 2A). After a booster immunization, a dramatic increase in the rate of seroconversion was observed for the group V rats (up to 80%). Only slight increases in the seroconversion rate (approximately 21 to 28%) were observed

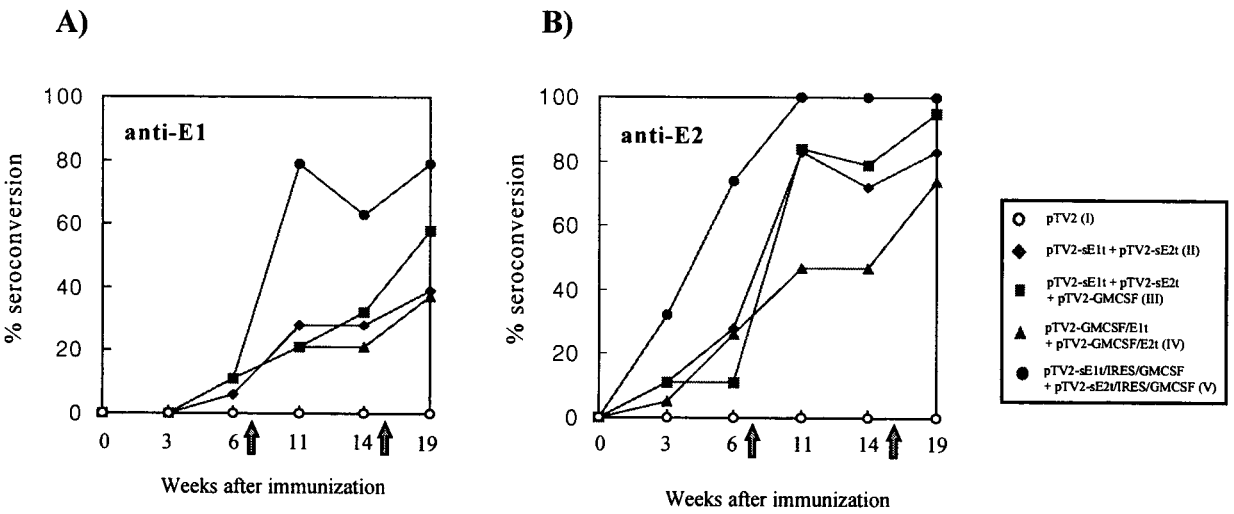

FIG. 2. Percent seroconversion of rats following plasmid injections. Female Buffalo rats were injected three times at 8-week intervals and anti-E1 (A) and anti-E2 (B) antibody responses were monitored as described in Materials and Methods. Arrows represent booster DNA injections at weeks 8 and 16. The different plasmids injected are summarized at the right.

for groups II, III, and IV. These rates were further enhanced by a second booster DNA injection at week 16. Final seroconversion rates of 39, 58, 37, and 79% were observed for groups II, III, IV, and V, respectively. In contrast to the responses of the anti-E1 antibody, anti-E2 antibody responses were detectable at week 3, indicating that anti-E2 antibodies were generated earlier than were anti-E1 antibodies in all the envelope DNA-immunized rat groups (Fig. 2B). Among these groups of rats, the group V rats that received bicistronic plasmids showed the highest seroconversion rate: approximately 70% at week 6 and 100% at week 8 when the initial booster DNA was administered. Seroconversion rates of 10 and 30% were observed at week 6 for the group II and the group III rats, respectively, but dramatic increases in seroconversion rates (up to 80%) were observed for both groups soon after the initial booster DNA injection at week 8. A second booster DNA injection at week 16 led to a slight increase in the seroconversion rates for the E2 protein in both groups. In contrast to other groups of rats, the group IV rats that received fusion plasmids had a seroconversion rate of 25% at week 6; this rate gradually increased to approximately 70% upon the administration of two booster DNA injections. These observations that the seroconversion rates were increased by booster DNA injections suggest that primed immune responses appear to be generated and amplified, a process which leads to rapid antibody responses after booster DNA immunizations.

In order to obtain a semiquantitative estimation of anti-E1 and anti-E2 antibody responses, we performed end point titrations by ELISA with serial dilutions of pooled sera obtained from seroconverted rats (18). As shown in Table 1, the antibody titers to HCV E1 and E2 proteins reached 442 and 5,662, respectively, in the group II rats. Slightly higher antibody titers were generated in groups III and IV rats by the codelivery of GM-CSF. As expected, the group V rats had the highest antibody titers to the E1 and the E2 proteins (1,615 and 71,647, respectively). Taken together, our results demonstrate that HCV envelope DNA-based immunization generated antibody responses to E1 and E2 proteins and that the E2t protein was more immunogenic than was the E1t protein. In addition, we found that the codelivery of the GM-CSF gene from plasmids that encoded HCV envelope proteins could modulate antibody responses. The coexpression of GM-CSF and E1t or E2t from a bicistronic plasmid was the most effective delivery method for the enhancement of antibody responses to HCV envelope proteins.

To determine which subclass of anti-E2 immunoglobulin G (IgG) isotypes was induced by HCV E2 DNA-based immunizations with the codelivery of GM-CSF, sera obtained at week 19 were also tested by using horseradish peroxidase-conjugated sheep anti-rat IgG1 (1:500) or IgG2a (1:2,000) secondary antibodies (Serotec). The E2 DNA-based immunizations appeared to predominantly produce IgG2a responses to the E2 protein in all the envelope DNA-immunized rat groups (data not shown). These observations are in agreement with other reports which have shown that intramuscular DNA immunizations preferentially elicited Th-1 type immune responses (9, 23).

Antibody responses to HVR1 peptides. To generate protective immunity against HCV infection, HCV envelope DNA-based immunizations must induce antibodies which are capable of neutralizing viral infection. Due to the lack of an effective in vitro cell culture system for the propagation of HCV, it is difficult to test the neutralizing capability of sera obtained from DNA-immunized rats. It has been previously reported that antibodies directed against hypervariable region 1 (HVR1) of the E2 protein could have neutralizing capability (38). We tested the ability of sera obtained from rats that were injected with GM-CSF and HCV envelope DNA to bind to HVR1 peptides. Various HVR1 peptides (Fig. 3A), including type 1b (HCV-K and HCV-J4; Korean and Japanese isolates, respectively) and type 1a (HCV-H), were purchased from the PeptidoGenic Research Co. (Livermore, Calif.). HVR1-specific antibodies were analyzed with serum samples from DNA-immunized rats by ELISA. Briefly, different HVR1 peptides (2 μg/ml) which were biotinylated at their N termini were coated overnight at 4°C following precoating treatment with streptavidin (2 μg/ml) for 2 h at room temperature. After a blocking with bovine serum albumin for 1 h, 100 μl of test sera (1:100 dilution) was added to each well and incubated for 1 h at room temperature. Bound antibodies directed at the HVR1 peptide were also detected with horseradish peroxidase-conjugated sheep anti-rat IgG (1:3,000 dilution) antibodies. Compared with that from the pTV2-immunized control group, sera from groups II, III, and V rats showed the ability to bind strongly to a homologous (HCV-K; type 1b) HVR1 peptide (amino acids [aa] 384 to 403) (Fig. 3B). In contrast to what was found for the total anti-E2 antibody titers, the levels of antibody responses to HVR1 were similar for groups II, III, and V, which indicated that GM-CSF codelivery had little effect on the generation of antibody responses to HVR1. The lower level of binding affinity of sera from GM-CSF/E2t DNA-immunized rats (group IV) may be due to a block of the HVR1 epitope by its N-terminal fusion with the GM-CSF protein. We also examined whether anti-HVR1 antibodies obtained from DNA-immunized rats had cross-reactivity to HVR1 peptides of heterologous strains. It was previously reported that isolate-independent anti-HVR1 antibodies seemed to map to the C terminus of HVR1 (39). Therefore, we tested the abilities of sera obtained from group I and group V rats to bind to various HVR1 peptides which did (aa 384 to 410) or did not (aa 384 to 403) express the C-terminal end of HVR1. Cross-reactive antibodies which were capable of binding to heterologous HVR1 peptides were generated by the HCV envelope DNA immunizations. Interestingly, heterologous HVR1 peptides which expressed the C-terminal end of HVR1 (aa 384 to 410) cross-reacted with the rat sera (Fig. 3C). However, heterologous HVR1 peptides that lacked this region (aa 384 to 403) did not cross-react or cross-reacted only slightly (data not shown). In addition, HVR1 expressed by the same genotype (HCV-J4; type 1b) reacted more strongly than did HVR1 expressed by a different genotype (HCV-H; type 1a). This is likely due to the higher degree of conservation of the C terminus of HVR1 between HCV-K and HCV-J4 (Fig. 3A). Sera from other groups of rats showed similar abilities to bind to heterologous HVR1 peptides, although the relative values of optical density at 405 nm for each group of rats varied. These results strongly suggest that HCV envelope DNA-based immunizations can generate cross-reactive antibodies to the HVR1s of various HCV strains.

Enhancement of lymphoproliferative responses by the codelivery of the GM-CSF gene. To investigate the effect of GM-CSF codelivery on the induction of the cellular immune response, the lymphocyte proliferative assay was performed as described previously (17). At 2 or 3 weeks after the final DNA inoculation, splenocytes were tested for their proliferation in response to stimulation with specific antigens. The isolated spleen cells were resuspended to a concentration of 3×10^6 cells/ml. A 100-μl aliquot was added to each well of a 96-well microtiter round-bottomed plate. Recombinant proteins were added to the wells in triplicate at a final concentration of 1 or 10 μg/ml. To assure that the spleen cells were healthy, concanavalin A (5 μg/ml) was used as a positive mitogenic control.

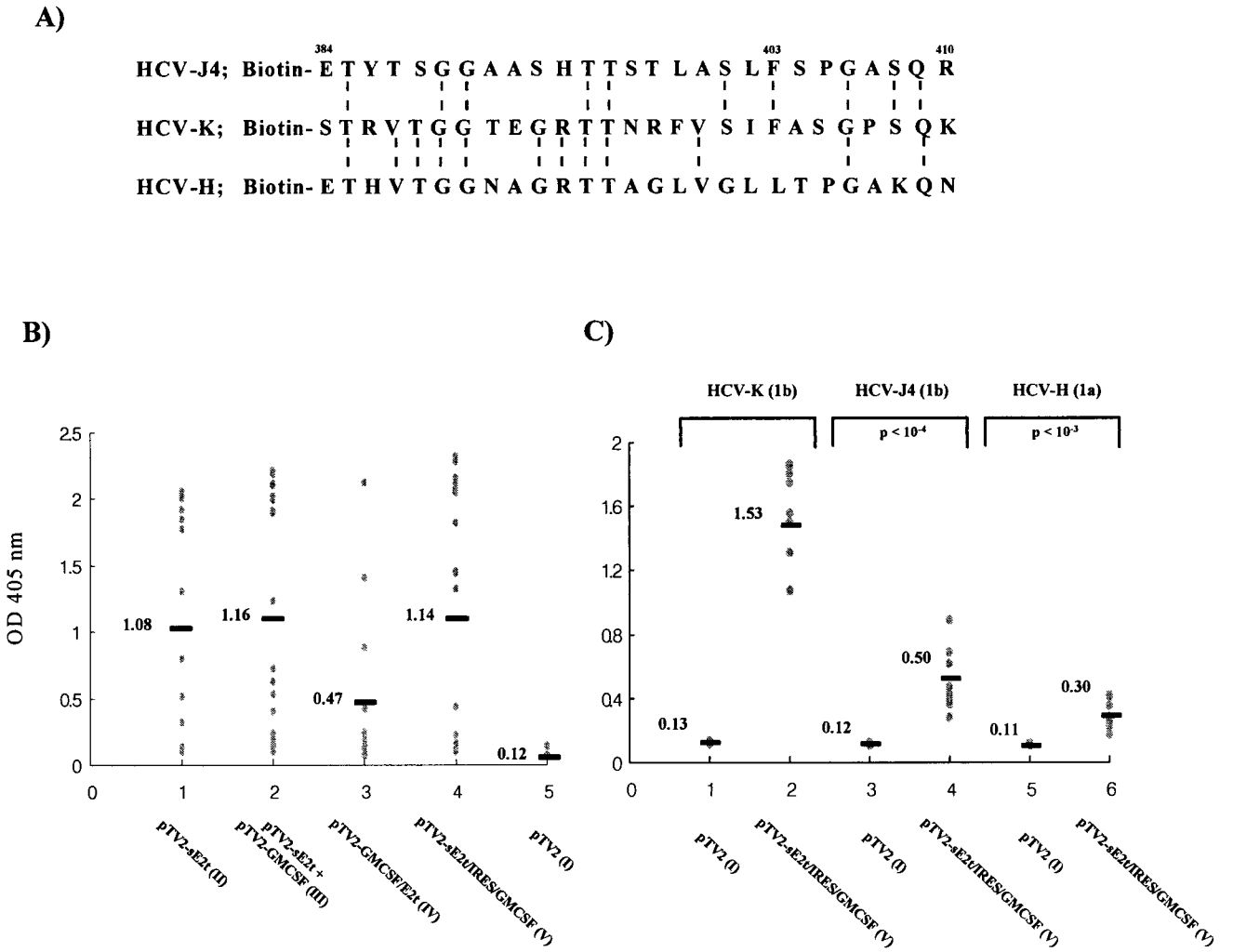

FIG. 3. Antibody responses directed to homologous and heterologous HVR1 peptides. Various HVR1 peptides which were biotinylated at their N termini were used in an ELISA. Sera bled at week 19 (1:100 dilution) were examined. In panels B and C, the optical density (OD) value at 405 nm for each seroconverted rat in the group is shown; average values are indicated by horizontal lines. The different plasmids injected are indicated at the bottom. (A) Comparison of various HVR1 sequences of different HCV strains. Amino acids that are the same for different HVR1 peptides are indicated by dashed lines, and the numbers of amino acids in the HCV polyprotein are indicated. (B) Abilities of sera obtained from DNA-immunized rats to bind to homologous HVR1 (HCV-K) peptide (aa 384 to 403). (C) Abilities of sera to bind to homologous and heterologous HVR1 peptides (aa 384 to 410). Statistical analysis was performed by the Student *t* test.

Lymphocytes from all rat groups did not have specific proliferative responses when a human growth hormone (hgh) protein, a negative control antigen, was used (Table 1). Spleen cells obtained from the group II rats had stimulation indices of approximately 4.4 and 9.8 with the addition of hghE2t and hghE1t proteins, respectively. When codelivered with the GM-CSF gene, HCV envelope DNA elicited higher lymphoproliferative responses to HCV envelope proteins than it did when administered without the GM-CSF gene to group II rats. These responses increased in a dose-dependent manner, and the peak stimulation index was approximately 6.3 to 7.0 for hghE2t and 13.0 to 11.9 for hghE1t for both group III and IV rats. However, a lymphoproliferative response was not detected by the addition of the hghE2t protein in the group I rats that received vector DNA alone. Although there were measurable lymphoproliferative responses to hghE1t in the group I rats, the responses were not dose dependent, which indicated that they were not antigen specific. Interestingly, the bicistronic coexpression of GM-CSF and HCV envelope proteins significantly enhanced lymphoproliferative responses to both hghE1t and hghE2t proteins, which reached stimulation indices of 14.7 and 11.2, respectively. These results indicate that the codelivery of the GM-CSF gene enhances T-helper cell responses to HCV envelope proteins in DNA-based immunization.

Conclusions. In this study, we described several observations concerning DNA immunizations using HCV envelope expression plasmids with or without the use of GM-CSF as a molecular adjuvant. For example, the immunogenic potential of the HCV E2t protein is likely to be higher than that of the HCV E1t protein, at least in our experimental conditions. This observation is compatible with the results of Lanford et al. (15), who observed a lower level of reactivity of anti-HCV-positive human sera to insect cell-expressed E1 protein (4 of 18 sera positive) than to analogously expressed E2 protein (15 of 18 sera positive). In addition, we have observed that both the humoral and the cellular immune responses to HCV envelope proteins were augmented by the codelivery of the GM-CSF

gene. The antibody and lymphoproliferative responses to the E2 protein were increased approximately 1.5- to 12.7-fold and 1.4- to 2.5-fold, respectively, by the codelivery of the GM-CSF gene. Inoculations of bicistronic plasmids elicited higher levels of antibody and lymphoproliferative responses than did the coinoculation of two independent expression plasmids that encoded the GM-CSF gene and each HCV envelope gene. These data suggest that the coexpression of GM-CSF and the envelope proteins from the same plasmid may optimize immune responses to the HCV envelope proteins. We suggest that the local concentration of GM-CSF may be one of the critical factors that contribute to the augmentation of immune responses to the coexpressed antigens. This model is partially compatible with the findings of Xiang et al. (36), who observed that the separate inoculation of the GM-CSF gene and antigen-encoding plasmids several hours apart had no effect on the magnitude of antigen-specific antibody responses. In addition, immunization with GM-CSF–envelope fusion constructs appeared to induce smaller immune responses than did those with bicistronic constructs. It is possible that the biological activity of GM-CSF may be altered when it is fused to the HCV envelope proteins. We conclude, therefore, that the bicistronic coexpression of GM-CSF and antigens is the most effective way to induce immune responses for the HCV envelope DNA-based immunizations.

It has been suggested that the HVR1 of the HCV E2 protein is comparable to the V3 loop of human immunodeficiency virus type 1 which contains a neutralizing determinant (22, 27, 34). Recently, it was shown that hyperimmune serum raised against a synthetic HVR1 peptide induced protection against homologous HCV infections in chimpanzees (7). In addition, the early appearance of antibodies that were directed against the N terminus of HVR1 is associated with acute self-limiting infections of HCV (39). Our experiments demonstrate that strong antibody responses to a homologous HVR1 peptide were induced in DNA-immunized rats. The levels of anti-HVR1 antibodies that were generated from groups II, III, and V rats were similar (Fig. 3B), which is likely due to the highly immunogenic potential of HVR1. In addition, we demonstrated that cross-reactive anti-HVR1 antibodies which mainly recognized the C-terminal end of the HVR1 were generated in DNA-immunized rats. These observations are consistent with clinical results that were obtained from acute and chronic HCV-infected patients. Cross-reactive antibodies directed to HVR1 have been observed during chronic HCV infection (29). These isolate-independent antibodies were shown to react to the C terminus of HVR1 (39). It is notable that HCV E2 DNA-based immunization can generate cross-reactive antibodies to heterologous HVR1, as is seen in natural infections of HCV. Although further studies concerning the neutralizing capability of these antibodies raised by DNA immunization are necessary, our studies suggest that immunization with plasmid DNAs that express HCV envelope proteins could result in the generation of protective immunity against heterologous HCV challenge.

S.W.L. and J.H.C. contributed equally to this work.

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